Original Article

Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii* from southern Brazil

Epidemiologia molecular de Acinetobacter baumannii resistente aos carbapenêmicos provenientes do sul do Brasil

Epidemiología molecular de Acinetobacter baumannii resistente a carbapenem del sur de Brasil

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RESUMO

Justificativa e Objetivos: a resistência aos carbapenêmicos em *Acinetobacter baumannii* atingiu níveis extremamente altos em todo o mundo, e as carbapenemases do tipo OXA classe D são o principal mecanismo associado. O objetivo deste estudo foi avaliar o perfil fenotípico e molecular de isolados clínicos de *A. baumannii* resistentes aos carbapenêmicos (CRAb) de uma região de fronteira do sul do Brasil. **Métodos:** a espécie *A. baumannii* foi identificada através da presença do gene $bla_{\text{OXA-51}}$, e o perfil de sensibilidade foi determinado por microdiluição em caldo. As principais carbapenemases foram investigadas por PCR, e a tipagem dos isolados de CRAb foi realizada por PFGE. **Resultados:** durante o período do estudo, 36 CRAb foram recuperados, dos quais 85,7% foram provenientes de amostras do trato respiratório de pacientes de UTI. Uma elevada resistência a aminoglicosídeos e fluoroquinolonas foi encontrada em contraste com 100% de sensibilidade a polimixina B. O gene $bla_{\text{OXA-23}}$ foi encontrado em 34 isolados e foi o único detectado além do $bla_{\text{OXA-51}}$. A tipagem molecular revelou a presença de quatro linhagens clonais, duas delas endêmicas ao longo do período do estudo. **Conclusão:** nosso estudo traz os primeiros dados sobre o perfil de resistência em *Acinetobacter* na fronteira oeste do sul do Brasil e alerta para a presença de clones endêmicos de CRAb produtores de OXA-23 nessa região, contribuindo para a construção do cenário epidemiológico nacional de CRAb.

Descritores: *Acinetobacter baumannii*. Carbapenêmicos. Resistência microbiana a medicamentos.

ABSTRACT

Background and Objectives: carbapenem resistance in *Acinetobacter baumannii* has reached extremely high levels worldwide, and class D OXA-type carbapenemases are the main associated mechanism. This study aimed to assess the phenotypic and molecular profile of clinical carbapenem-resistant *A. baumannii* (CRAb) isolates from a southern Brazilian border region. **Methods:** *A. baumannii* species was identified by the presence of the *bla*$_{\text{OXA-51}}$ gene, and the susceptibility profile was determined by broth microdilution. The main carbapenemases were investigated by PCR and the molecular typing was performed by PFGE. **Results:** during the study, a total of 36 CRAb were recovered, of which 85.7% were from respiratory tract samples from ICU patients. High level resistance to were found in contrast to 100% of susceptibility for polymyxin B. The *bla*$_{\text{OXA-23}}$ gene was present in 34 isolates and was the only one detected other than *bla*$_{\text{OXA-51}}$. Molecular typing revealed the presence of four clonal strains, two of them endemic during the period of the study. **Conclusion:** to the best of our knowledge, our study brings the first data about resistance profile in *Acinetobacter* in the western border of southern Brazil and make aware of endemic clones of CRAb-producing-OXA-23 in this region of state, contributing for the construction of the national epidemiologic scenario of CRAb.

Keywords: *Acinetobacter baumannii*. Carbapenems. Drug resistance, microbial.

RESUMEN

Justificación y Objetivos: la resistencia a carbapenémicos en *Acinetobacter baumannii* ha alcanzado niveles extremadamente altos en todo el mundo y las carbapenemases OXA de clase D son el principal mecanismo asociado. El objetivo de este estudio fue evaluar el perfil fenotípico y molecular de ~~los~~ aislados clínicos de *A. baumannii* resistentes a carbapenémicos (CRAb) de una región fronteriza en el sur de Brasil. **Métodos:** la especie *A. baumannii* se identificó a través de la presencia del gen *bla*$_{\text{OXA-51}}$ y el perfil de sensibilidad se determinó por microdilución en caldo. Las principales carbapenemasas fueron investigadas por PCR y la tipificación se hizo con PFGE. **Resultados:** durante el período de estudio, se recuperaron 36 CRAb, 85,7% de muestras del tracto respiratorio de pacientes de la UCI. Se encontró una alta resistencia a los aminoglucósidos y las fluoroquinolonas en contraste con 100% de sensibilidad a polimixina B. El gen *bla*$_{\text{OXA-23}}$ se encontró en 34 aislamientos y fue el único detectado además de *bla*$_{\text{OXA-51}}$. La tipificación molecular reveló la presencia de cuatro cepas clonales, dos de ellas endémicas durante el período de estudio. **Conclusiones:** hasta donde sabemos, nuestro estudio trae los primeros datos sobre el perfil de resistencia en *Acinetobacter* en la frontera oeste del sur de Brasil y reconoce los clones endémicos de CRAb productores de OXA-23 en esta región del estado, contribuyendo para la construcción del escenario epidemiológico nacional de CRAb.

Palabras-Clave: *Acinetobacter baumannii*. Carbapenémicos. Farmacorresistencia Microbiana

INTRODUCTION

Acinetobacter baumannii is a well-established pathogen worldwide that is responsible for several outbreaks and nosocomial infections with high levels of morbidity and mortality worldwide.[1-3] The *Acinetobacter* genus quickly acquired

resistance to several classes of antibiotics and the vast majority of beta-lactams, including broad-spectrum cephalosporins, and due to this, carbapenems have become the manly choice for the treatment of *Acinetobacter* infections in the last three decades.[1] However, isolates with multi-drug resistance (MDR) phenotype have become highly prevalent, and the emergence of mechanisms that confer resistance to carbapenems make treating infections a worrying challenge.[2]

The major mechanism responsible for carbapenem resistance in *A. baumannii* is due to OXA-carbapenemases and less frequently due to Class B carbapenemases.[1] The OXA enzymes commonly described in *A. baumannii* are divided in 6 subfamilies, comprising the intrinsic OXA-51-like and the acquired OXA-23-like, OXA-24-like, OXA-58-like, OXA-143 and OXA-235, all of them already reported in Brazil, except OXA-235.[4, 5] The *bla*$_{\text{OXA-23-like}}$ gene is the most frequently reported in clinical isolates of carbapenem-resistant *A. baumannii* (CRAb) worldwide.[1, 2] The first case reported in Brazil was in 2003, in Curitiba city, and since then, there was a nationwide spread.[4-6] In Brazil, the carbapenem resistance rate in *Acinetobacter* spp. reaches almost 80%, according to the Brazilian National Health Surveillance Agency[7], and OXA-23 producing CRAb is associated with several outbreaks and high mortality rates in ICUs.[8, 9]

Considering that data about bacterial resistance are still rare in some regions of the country, this study aimed to assess the phenotypic and molecular profile of clinical carbapenem-resistant *A. baumannii* (CRAb) isolates from a southern Brazilian border region.

METHODS

Bacterial isolates

A total of 39 non-duplicated clinical isolates of *Acinetobacter* spp., resistant or with reduced susceptibility to carbapenems by disk-diffusion method were selected. They were recovered from a bank of clinical isolates constituted as part of a surveillance study on carbapenem resistance in the western border of Rio Grande do Sul State, from May 2014 to December 2018, including gram-negative bacilli from community and hospital. The 39 isolates, previously identified as *Acinetobacter* spp., had their identification confirmed by conventional techniques (*coccobacilli* in Gram staining, catalase and oxidase reactions and non-fermentation on TSI agar), then they were submitted to PCR for *bla*$_{\text{OXA-51-like}}$ gene to identify *A. baumannii* isolates.[10] This study

was developed as part of a main project approved by Research Ethics Committee from *Universidade Federal do Pampa* (UNIPAMPA) (CAAE 32723414.2.0000.5323).

Susceptibility profile

Broth microdilution was performed for *A. baumannii* isolates to confirm the susceptibility profile to carbapenems. The isolates resistant to IPM and/or MER, designated as CRAb, were also assessed against ceftriaxone, ceftazidime, cefepime (CFP), amikacin (AMI), gentamicin (GEN), ciprofloxacin (CIP), and polymyxin B (POL). MICs were interpreted according to *Clinical and Laboratory Standards Institute* breakpoints.[11] *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

Carbapenemase detection

Carbapenemase-encoding genes, including the main OXA-types, were investigated by conventional PCR for CRAb, as previously described (Table 1). Primers are shown in Table 1. The amplified PCR products were visualized by electrophoresis on 1.5% (w/v) agarose gels in a 0.5 X tris-borate-EDTA buffer, stained with SYBR safe, using the photo documentation Alphalmager HP (ProteinSimple, USA) system. The *E. coli* ATCC strain 25922 was used as negative control.

Table 1. Sequence of primers used to detect carbapenemase-encoding genes

Target	Primer	Sequence (5' to 3')[1]	Amplicon size (bp)	Reference
bla$_{\text{OXA-51-like}}$	OXA-51f	TAA TGC TTT GAT CGG CCT TG	353	12
	OXA-51r	TGG ATT GCA CTT CAT CTT GG		
bla$_{\text{OXA-143}}$	OXA-143f	TGGCACTTTCAGCAGTTCCT	149	13
	OXA-143r	TAATCTTGAGGGGGCCAACC		
bla$_{\text{GES}}$	GESf	ATGCGCTTCATTCACGCAC	860	14
	GESr	CTATTTGTCCGTGCTCAGG		
bla$_{\text{OXA-23-like}}$	OXA-23f	CCC CGA GTC AGA TTG TTC AAG G	330	15
	OXA-23r	TAC GTC GCG CAA GTT CCT GA		
bla$_{\text{OXA-24-like}}$	OXA-24f	GCA GAA AGA AGT AAA RCG GGT	271	
	OXA-24r	CCA ACC WGT CAA CCA ACC TA		
bla$_{\text{KPC}}$	KPCf	TCG CCG TCT AGT TCT GCT GTC TTG	353	
	KPCr	ACA GCT CCG CCA CCG TCA T		
bla$_{\text{NDM}}$	NDMf	ACT TGG CCT TGC TGT CCT T	603	
	NDMr	CAT TAG CCG CTG CAT TGA T		
bla$_{\text{IMP}}$	IMPf	ACA YGG YTT RGT DGT KCT TG	387	
	IMPr	GGT TTA AYA AAR CAA CCA CC		

bla_{VIM}	VIMf	TGT CCG TGA TGG TGA TGA GT	437
	VIMr	ATT CAG CCA GAT CGG CAT C	
bla_{OXA-48}	OXA-48f	ATG CGT GTA TTA GCC TTA TCG	265
	OXA-48r	CAT CCT TAA CCA CGC CCA AAT C	

Genotyping

The genetic relationship among CRAb isolates were performed using an enzymatic restriction with *ApaI* (Thermo Scientific, USA) followed by pulse-field gel electrophoresis (PFGE), performed according to the Centers for Disease Control and Prevention (CDC) guidelines, with modifications.[16] Fragments were separated on 1.0% (w/v) agarose gel in a 0.5 X tris-borate-EDTA buffer for 23 h at 14°C using a pulse ramp rate changing from 5s to 35 s, at 6 V/cm in the CHEF-DRIII System (Bio-Rad, USA) apparatus. The restriction patterns were analyzed by GelJ software (version 2.0), with dice similarity coefficient and the unweighted-pair group method using average linkage (UPGMA) algorithm with 1.5 % band matching tolerance. Genetic and clonal relatedness were established for similarity values ≥ 85% and ≥ 99%, respectively.[17]

RESULTS

All the 39 isolates tested presented the bla_{OXA-51} gene; however, only 36 were confirmed as CRAb by broth microdilution. The majority of CRAb isolates were from clinical samples from ICU patients (*n=21*) isolated from respiratory tract specimens (*n=18*) (Figure 1). The isolates susceptibility profile is show in Figure 1. In addition to carbapenems, CRAb isolates were also resistant to third generation cephalosporins, cefepime and quinolones. All isolates were classified as MDR (non-susceptible to ≥1 agent in ≥3 antimicrobial categories).[18]

Molecular analysis revealed the presence of $bla_{OXA-23-like}$ gene in 34 CRAb. None of the other genes searched were detected among the isolates. *ApaI*-PFGE dendrogram of CRAb evidenced the presence of two clusters (I and II) and three singletons. The main cluster (I) included 17 isolates distributed from 2014 to 2018 and one main clonal lineages strains, type A (*n=14*). Cluster II included 13 isolates and two clonal lineages, B and B1, that appeared more recently, in 2017. In cluster III (*n=3*) only isolates collected in 2016 were present. Two of the three singletons represented the isolates 1ST and 16A, both non-carriers of the $bla_{OXA-23-like}$ gene (Figure 1).

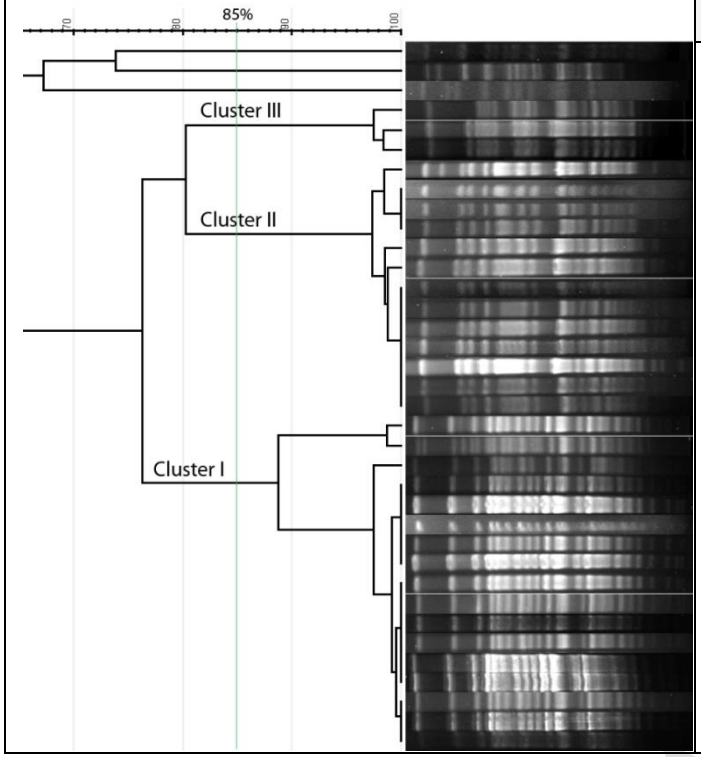
DISCUSSION

Ventilator-associated pneumonia (VAP) is the most frequently acquired infection in ICUs, with incidence rate ranging from 5% to 67%, and *A. baumannii* recovered from endotracheal aspirates and bronchoalveolar lavage is one of the most prevalent pathogens associated to VAP often involving MDR strains.[19] In the present study, 85,7% (16/21) of ICU isolates were recovered from respiratory tract samples. Data of a national study involving isolates provided from endotracheal aspirates isolates from ICU patients also demonstrated a high prevalence (70.6%/ *n=29*) of OXA-23-producing CRAb associated to VAP.[20]

Although all of the CRAb isolates assessed in this study was MDR, all of them were susceptible to polymyxin B. Polymyxins play an important role in the treatment of CRAb infections due to the limited therapeutic options available.[1] Despite the low MIC values (≤1.0 μg/mL) found to POL in this study, it is quite likely that this profile may change in a next future as a consequence of its increased therapeutic use in recent years. Aminoglycosides have proved to be a great therapeutic option when susceptibility permits[1,2]; however, high-level resistance to these drugs were also observed among CRAb, with only five isolates sensitive to gentamicin and two to amikacin. Indeed, but due to the toxicity in prolonged uses, they are commonly combined with other antimicrobials such as colistin and β-lactams to treat MDR *A. baumannii*.[2, 3] In recent years, different resistance mechanisms to these drugs have emerged leading to a considerable decrease in susceptibility in *A. baumannii* isolates worldwide.[21]

The susceptibility profile also revealed a considerable increase in MIC values over the years, mainly for β-lactams. It could be related to an overexpression of intrinsic genes like bla_{ADC}, responsible for conferring natural resistance to narrow-spectrum cephalosporins.[22] Although broad-spectrum cephalosporins are not hydrolyzed by class D carbapenemases, ceftazidime and cefotaxime resistance in OXA-23-producing *A. baumannii* isolates were assigned to AmpC overproduction. The association of other mechanisms, such as the overexpression of *adeABC* efflux systems should also be considered, as they can contribute for the increasing meropenem and ceftazidime MICs.[23]

Thus, $bla_{\text{OXA-23-like}}$ gene was responsible for resistance phenotype found in 94.4% of CRAb isolates. In 2014, a study performed in the capital of state, 600 km far from the western border, showed that OXA-23 was the main resistance mechanism associated to CRAb, remaining widespread five years after the first outbreak in the city.[24]



ApaI-PFGE type	Isolate	Clinical Specimien	Year of collection	MIC (µg/mL) [b]								
				CRO (R ≥ 64)	CAZ (R ≥ 32)	CPM (R ≥ 32)	IPM (R ≥ 8)	MER (R ≥ 8)	AMI (R ≥ 64)	GEN (R ≥ 16)	CIP (R ≥ 4)	POL (R ≥ 4)
E	16A*	Blood	2017	>256	128	32	16	32	64	4	64	0,5
D	1ST	Catheter	2014	>256	128	32	16	1	256	>256	>64	0,5
F	30A*	OTS[a]	2018	128	32	>256	32	256	256	32	>256	0,5
C2	3A	Wound secretion	2016	>256	>256	32	8	32	128	2	32	0,5
C1	2A	Wound secretion	2016	>256	>256	32	8	32	128	2	32	1
C	1A	BAL	2016	>256	>256	32	8	16	64	1	32	0,5
B1	24A*	OTS[a]	2018	>256	128	>256	16	64	128	16	>256	0,5
B	35A*	OTS[a]	2018	>256	>256	>256	16	128	256	16	>256	0,5
	31A*	Tracheal Secretion[a]	2018	>256	128	128	16	64	256	16	>256	0,5
	17A*	OTS	2017	>256	64	32	16	32	64	2	32	0,5
	13A	Wound secretion	2017	>256	128	32	16	64	64	256	64	1
	9A*	OTS[a]	2017	>256	128	32	16	32	32	256	64	0,5
	21A*	OTS[a]	2018	>256	128	256	32	128	256	32	16	0,5
	19A*	Catheter[a]	2018	>256	128	32	16	32	32	256	32	0,5
	14A	OTS	2017	>256	128	32	16	64	64	256	64	0,5
	26A*	OTS[a]	2018	>256	128	>256	32	64	128	>256	>256	0,25
	25A*	OTS[a]	2018	>256	256	>256	32	128	128	>256	>256	0,5
	18A*	Tracheal Secretion[a]	2017	>256	128	32	16	32	32	128	32	0,5
	5A*	OTS[a]	2017	>256	64	16	16	32	64	256	64	0,25
A3	9ST	OTS	2014	>256	256	16	32	64	32	>256	>64	0,5
A2	8ST	Wound secretion	2014	>256	256	32	16	32	32	>256	>64	0,5
A1	19ST	Wound secretion	2015	>256	32	64	16	8	2	128	64	0,5
	21ST	OTS	2015	>256	256	32	32	64	32	>256	64	0,5
	12ST*	BAL[a]	2014	>256	128	32	32	64	32	>256	32	0,5
	33A	Sputum	2018	>256	128	128	16	128	128	>256	>256	0,25
	6ST*	Cerebrospinal fluid	2014	>256	128	32	16	64	32	>256	32	0,5
	3ST*	OTS[a]	2014	>256	256	64	32	32	32	>256	16	1
	2ST	OTS	2014	>256	256	64	32	64	32	>256	32	0,5
A	27A	Wound secretion	2018	>256	128	>256	16	64	64	>256	>256	0,5
	20A*	Urine	2018	>256	128	>256	16	64	128	>256	>256	0,5
	28A*	Sputum[a]	2018	>256	256	>256	16	64	128	>256	>256	0,5
	16ST*	BAL[a]	2014	>256	128	32	8	8	2	>256	>64	0,5
	13ST	OTS	2015	>256	128	32	128	32	64	>256	32	0,5
	29A	OTS	2018	>256	128	>256	16	64	64	>256	>256	0,25
	11ST*	Catheter[a]	2014	>256	256	16	32	32	32	>256	32	0,5
	8A*	OTS[a]	2017	>256	128	16	32	64	32	>256	64	0,5

Figure 1. Molecular typing and phenotype profile of CRAb isolates

Caption:
Typing obtained by *Apa*I-PFG digestion. Dendrogram displaying the genetic relatedness for all $bla_{\text{OXA-51-like}}$ gene producing isolate constructed using Dice coeficient with 1,5 % band matching tolerance and UPGMA for clustering.
*ICU patients.
OTS: Orotracheal tube secretion; BAL: Bronchoalveolar lavage.
[a] Respiratory tract sample provided from ICU patients.
[b] Resistance MIC interpretation according to CLSI 2018 [11]. AMI: Amicacin; CIP: Ciprofloxacin; CPM: Cefepime; GEN: Gentamicin; IPM: Imipenen; MER: Meropenem; POL: Polymixin B.

In a recent study including CRAb clinical isolates from four Brazilian states, 87% (*n=80*) were positive for the presence of the *bla*$_{\text{OXA-23-like}}$ gene.[25] Other studies confirm a high prevalence of OXA-23-producing CRAb associated with high mortality rates in ICUs in different Brazilian cities.[8, 9]

16A and 1ST isolates were negative for the presence of all carbapenemase-encoding genes screened, except for *bla*$_{\text{OXA-51-like}}$. A study has shown that this gene, besides to be constitutive of the species, may be related to high MICs for carbapenems depending on the presence of the insertion elements, as IS*Aba*1.[22]

Outbreaks due to CRAb in this hospital have occurred since 2014, when we initiated the epidemiologic surveillance study of carbapenem resistant in gram-negative *bacilli*. Our data evidenced the prevalence of a major clonal lineages (type A) as well as the emergence of new ones (type B and C), since 2016. Several studies have described outbreaks caused by a single clone in a same institution although polyclonal outbreaks are not rare.[26] Until the moment, in 2018 was registered the highest number of cases of CRAb in the last five years. *A. baumannii* has the ability to survive on environmental surfaces for long periods, making transmission difficult to control. This feature is directly associated to hospital outbreaks.[2,3] For a more dynamic and comprehensive epidemiological understanding, it would be interesting to carry out typing by MLST, in order to check whether the clones found are endemic in Brazil or even Latin America or whether represent a distinct and peculiar profile of the border region.

In conclusion, our results provide the first data on the local epidemiology of *Acinetobacter* resistance, evidencing the spread and permanence of OXA-23-producing *A. baumannii* with high level resistance to β-lactam, quinolones and aminoglycosides. The genetic typing revealed the permanence of two endemic lineages clonally related in the hospital, followed by the spread of polyclonal strains, highlighting that CRAb is a worrisome challenge not only restricted to large heath centers. Thereby, the understanding of resistance mechanisms and local epidemiology provide important tool, in order to improve the appropriate treatment for serious infections, contributing for control and prevention of infections caused by CRAb.

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